AD_____

Award Number: W81XWH-07-1-0228

TITLE: Developing a Zebrafish Model of NF1 for Structure-Function Analysis and Identification of Modifier Genes

PRINCIPAL INVESTIGATOR: Jonathan A. Epstein, M.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

REPORT DATE: April 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30-04-2008	Annual	1 APR 2007 - 31 MAR 2008

4. TITLE AND SUBTITLE

Developing a Zebrafish Model of NF1 for Structure-Function Analysis and Identification of Modifier Genes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0228

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jonathan A. Epstein, M.D.

Email: epsteinj@mail.med.upenn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pennsylvania
Philadelphia, PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This progress report summarizes the first year of activity of this project focused on the identification and characterization of the zebrafish orthologs of the neurofibromatosis type 1 genes. This project involves work within the Epstein laboratory and collaboration with the laboratory of Dr. Thomas Look at the Dana Farber Cancer Institute as a sub-contract. This progress report summarized the collaborative work including results from both groups. During the first year, significant progress has been made in the isolation and characterization of two zebrafish orthologs, znf1a and znf1b. The genes have been sequenced and their expression patterns identified. The knockdown phenotypes have been characterized and include overgrowth of glia and cardiovascular abnormalities. Work towards identification of stable mutant fish lines has begun. Manuscripts are in preparation.

15. SUBJECT TERMS
Zebrafish model of neurofibromatosis; Morpholino knockdown; Engineered zinc fingered nuclease; Tilling; Vascular Patterning;

E d di l C hi Cli

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION:

The purpose of this project is to develop a zebrafish model of type 1 neurofibromatosis. The reason that this animal model may be useful for the further understanding of this disease is that genetic and chemical screens will be possible in a high throughput format using this vertebrate system. In addition, structure-function analysis will be possible using the developing zebrafish model. Prior to this work, the Nf1 genes in zebrafish had not been described or characterized. Zebrafish develop rapidly and are transparent during development allowing for easy visualization of many structures including the cardiovascular system. Genetic and chemical screens can be performed in high throughput modalities at relatively low cost. Emerging technologies allow for complex manipulation of gene expression. This project involves the collaboration of two established groups to coordinate efforts to take advantage of zebrafish as a model system for Nf1.

BODY:

Designing splice-blocking morpholinos against zNf1a and zNf1b

We have designed splice-blocking morpholinos that transiently block the function of zNf1 genes by preventing normal splicing of the transcripts. At present, there are two working morpholinos against zNf1a, namely zNf1a-SBe1 and zNf1a-SBe7, and one morpholino against zNf1b, namely zNf1b-e4. Figure 1A and 1B show the location of the morpholinos relative to the genomic structures of zNf1 genes. RT-PCR at 3dpf (days postfertilization) shows that all morpholinos are able to block the normal splicing efficiently (Fig. 1C and 1D, upper panels, and data now shown). When the aberrant lower bands upon morpholino knockdown were sequenced, the whole or part of exon targeted was confirmed to be missing as the result of forced utilization of the wrong or cryptic splicing donors upon morpholino injections as diagramed in Fig. 1A and 1B. We also sequenced some of the aberrant upper bands in zNf1a-e7 knockdown, and they retain introns that lead to the early stop for translation. The efficacy of zNf1b-e4 was further tested quantitatively by real time RT-PCR, showing almost 85% knockdown (Fig. 1E).

One problem we encountered during experiment was that when two morpholinos against zNf1a and zNf1b were injected together to knock down two genes simultaneously, there were significant necroses in the early embryos, especially in the head region, due to the non-specific toxicity of the morpholinos that makes it impossible to analyze the phenotypes correctly. Therefore, to circumvent the problem, we adopted a strategy of injecting morpholinos into p53 homozygous mutant background since it is known that the non-specific toxicity is largely mediated by p53 pathway. In fact, we were able to get rid of the head necrosis by using this strategt almost completely (Fig. 1F), and the following experiments were mostly carried out in the p53 mutant background.

The number of OPCs are increased upon zNf1 knockdown

Nf1 patients often show deficits in learning and structural abnormality in the brain such as macrocephaly due to the increased white matter, and the role or Nf1 in glial development has been studied in cell culture and mouse models. In order to see whether zebrafish can be used to study the role of Nf1 in glial development, we have blocked the Nf1 function using our morpholinos into olig2-EGFP transgenic zebrafish with wild type or p53 mutant in which GFP labels oligodendrocyte precursor cells (OPCs) and oligodendrocytes during embryogenesis. In olig2-EGFP transgenic zebrafish, some OPCs that are born at the ventral spinal cord during early embryogenesis migrate toward to the dorsal spinal

cord, while others stay in the ventral domain. As they differentiate into oligodendrocytes they send out processes that eventually myelinate nearby axons.

In morpholino-injected transgenic embryos, we have consistently found increased number of OPCs at 3dpf in most of our injected conditions based on the number of migrating OPCs (Fig. 2). The most dramatic increase, however, was found in combined blocking of zNf1a and zNf1b in p53 mutant background (Fig. 2C), compared to uninjected p53 mutant or wild type olig2-EGFP fish (Fig. 2A, 2B), suggesting both of these genes contributing the OPC increase. This phenotype was validated by co-immunostaining with sox10 antibody (a gift from Appel lab at Vanderbilt University) that labels only OPCs throughout the spinal cord at this stage; this double staining is necessary because the motor neurons in the ventral domain are also positive with GFP. The increased number of double positive cells for both GFP and sox10 in zNf1a and 1b knockdown in p53 mutant were also evident in migrating OPCs as well as in the ventral spinal cord in sections (Fig. 2D-F). This difference was verified statistically significant by comparing the numbers of double-positive cells (36% increase of OPC number in zNf1a+1b in p53 mutant compared to uninjected p53 mutant; Fig. 2G). This phenotype upon zNf1 knockdown strongly suggests that Nf1 plays an important role in OPC development consistent with the previous studies. We believe this phenotype is mostly due to the loss of zNf1 function because (1) blocking zNf1a function alone in the wildtype olig2-EGFP transgenic fish also causes the increased number of OPC significantly (Fig. 2G), and (2) there is only minor increase in uninjected p53 mutant compared to the uninjected wild type. However, we cannot completely rule out the possibility of the synergistic effect of the simultaneous loss of zNf1 and p53 at the moment.

MAPK signaling appears to be upregulated upon zNf1 knockdown, but not in OPCs

In order to address whether Ras pathway is affected upon zNf1 knockdown and responsible for OPC increase, the phospho ERK (pERK) antibody that recognizes phosphorylated form of ERK 42/44 as a readout for one of the downstream targets for Ras were immunostained. It appears that ERK pathway is activated upon knockdown based on the fact that larger populations of cells are labeled with pERK antibody (Fig. 3A and 3B). Interestingly, however, the GFP-positive OPCs are never co-labeled with pERK antibody throughout the sections (arrows; n=5). This suggests that the OPC phenotype is either independent of pERK signaling or requires it non-cell autonomously.

Cell proliferation and cell death are not evident in morphants by pH3 staining and TUNEL

In order to see whether cell proliferation or cell death accounts for the increased number of OPCs, phospho-histone H3 (pH3) antibody that labels the dividing cells or TUNEL staining that labels cells undergoing apoptosis were applied to sections at the 3dpf spinal cord. Either pH3- or TUNEL-positive cells were very rarely found in the uninjected or morpholino injected embryos (Fig. 4A-D), suggesting that at least active proliferation or reduction of cell death at this stage is not the main cause of the increased OPC phenotype. We plan to do similar experiments at earlier time points to see if an earlier defect in these phenomena contributes to the phenotypes during later development.

Cardiovascular defects in zNf1 knockdown zebrafish

zNf1a and zNf1b knockdown embryos also displayed gross abnormalities of cardiovascular development appreciable to the blinded observer by 48 hpf (Figure 5A,B). A majority of knockdown embryos displayed large pericardial effusions and frequently a “to-and-fro” movement of blood was apparent within the heart. That is, blood was seen to move back and forth from atrium to ventricle, suggesting a malfunctioning atrio-ventricular valve. Overall development of the embryos was relatively preserved through the first 72 hours of development despite the cardiac defects. Histologic analysis

revealed thinned ventricular myocardium and large pericardial effusions (not shown). Non-specific toxicity from MO exposure as a cause of the observed cardiovascular defects was unlikely since unrelated control or scrambled MOs did not produce similar levels of cardiovascular abnormalities, defects were observed even at low doses of specific MOs, and similar defects were seen with several unrelated but specific MOs against zNf1a and zNf1b. In addition, injection of specific MOs in p53 mutant embryos also produced identical cardiovascular defects, and non-specific toxicity due to MO exposure is thought to be at least partially p53-dependent. Pericardial effusion, cardiac valve defects and thinned myocardium are also seen in murine embryos deficient in Nf1.

We performed knockdown experiments in zebrafish embryos in which endothelial cells are marked by expression of EGFP in order to allow for more detailed analysis of vascular development (Figure 5C,D). At 24 hpf, we noted subtle abnormalities of vascular patterning in the intersomitic vessels which became more dramatic by 48 and 72 hpf. The leading edge of sprouting vessels had claw-like projections and failed to pattern normally such that the dorsal vessel failed to form or developed in only a rudimentary fashion. This occurred in embryos that were otherwise normal in size and overall maturity. Vascular patterning defects did not appear to correlate directly with cardiac defects, as we observed embryos with vascular abnormalities that did not display pericardial effusion or "to-and-fro" movement of blood within the heart. Blood flow within the dorsal vessel appeared intact in these embryos.

Vascular patterning defects in mouse embryos lacking Nf1

Although cardiac defects have been reported in mouse embryos lacking Nf1, and this has been attributed to a role for neurofibromin in endothelium, vascular patterning defects have not been previously identified. However, murine knockouts succumb during mid-gestation and exhibit significant peripheral hemorrhage that has been attributed to cardiac failure. In light of our observations of peripheral vascular pattering defects in zebrafish knockdown embryos, we re-examined murine Nf1 mutants using whole mount PECAM staining to visualize endothelium. We identified significant vascular abnormalities in these embryos. These findings indicate that a previously unrecognized phenotype in the mouse mutant was identified based upon observations derived from the zebrafish disease model.

Identifying zNf1a and zNf1b mutant lines

We are trying to identify mutants for zNf1a and zNf1b by adopting several ways. First, we have been using TILLING (targeting-induced local lesions in genomes) method, which screens the mutagenized zebrafish genomic DNA library by direct sequencing or CelI enzyme digestion (Fig. 6A). We have sequenced the mutagenized library from Dr. Moens at Univ. of Washington, and found one potential mutant with a missense mutation for zNf1a (Fig. 6B; #1). We are also collaborating with Dr. Solnica-Krezel laboratory at Vanderbilt University for TILLING zNf1a (targeting-induced local lesions in genomes), and found four possible missense mutations so far (Fig. 6B; #2~5). We are in the middle of outcrossing these founder fish for at least a few generations in order to expand these lines and remove the background mutations, especially with olig2-EGFP transgenic fish so that we can directly assess the OPC phenotype in details. We will investigate the phenotype in the mutant alone for zNf1a or along with the morpholino injection against zNf1b in case the OPC phenotype is obvious only when two genes are non-functional together.

Second, we take advantage of the resources from Znomics, who have generated a library of mutant fish with the retrovirus insertions. We have identified two potential insertional mutants for zNf1b from its library (Fig. 6C). At present, we have recovered one of the lines and will assess the phenotype

similar to zNf1a potential mutants. Finally, we have begun a collaboration with Nathan Lawson at UMass who has recently demonstrated that engineered zinc finger nucleases can be employed to introduce germline mutations into the genome of fish. We have begun experiments to mutate zNf1a and zNf1b using this approach.

In summary

- The number of OPCs in the spinal cord are increased upon transient knockdown of zNf1a and zNf1b
- Phospho MAP kinase pathway may not be responsible for the OPC increase or be required non-cell autonomously
- No obvious increase of cell division or decrease of cell death is detected upon MO injections
- Cardiovascular defects in zNf1 knockdown fish
- Several putative mutants for zNf1a and zNf1b are being identified and validated

Materials and Methods

1. **Immunohistochemistry** Fixed embryos were embedded in sucrose/agar mix and cryosectioned using cryostat machine with 14μm thick. Sections were incubated with the primary antibodies (sox10, 1:5,000; GFP, 1:500, pMAPK, 1:200, pH3, 1:200) and Alexa 568 or 633 secondary antibodies.
2. **Whole mount in situ hybridization** Fixed embryos were permeabilized with proteinase K treatment (10ug/ml) with various incubation time depending on the stages of embryos, which then were incubated with DIG-labeled antisense RNA probes and visualized using alkaline phosphatase-conjugated anti-DIG antibody in color substrates.
3. **RT-PCR** cDNA for RT-PCR was made by reverse transcribing RNA prepared from morpholino (MO) injected embryos using Trizol, and PCR was performed to detect potential aberrant splicing. The triplicates of the same cDNA were used for the quantitative PCR to measure the knockdown by zNf1b-e4 with beta-actin as baseline control.
4. **Confocal imaging** Zeiss LSM 510 META confocal microscope at Harvard Neurodiscovery imaging program was used for capturing confocal images of olig2-EGFP and other antibody staining in zebrafish spinal cord with 1-3μm optical sections.
5. **Counting OPCs and statistical analysis** Three sections of the spinal cord at the approximately similar anterior-posterior level from individual embryos (eight to ten embryos) were chosen for counting all of the GFP+/sox10+ cells within the section. Individual t-test were performed to test the significant differences in the number of GFP+/sox10+ cells in the spinal cord using Prism software.
6. **TUNEL** Chemicon ApopTag Red In Situ Apoptosis Detection Kit (S7165) was used for TUNEL staining. The protocol for cryosections in the munual was followed with minor modifications.

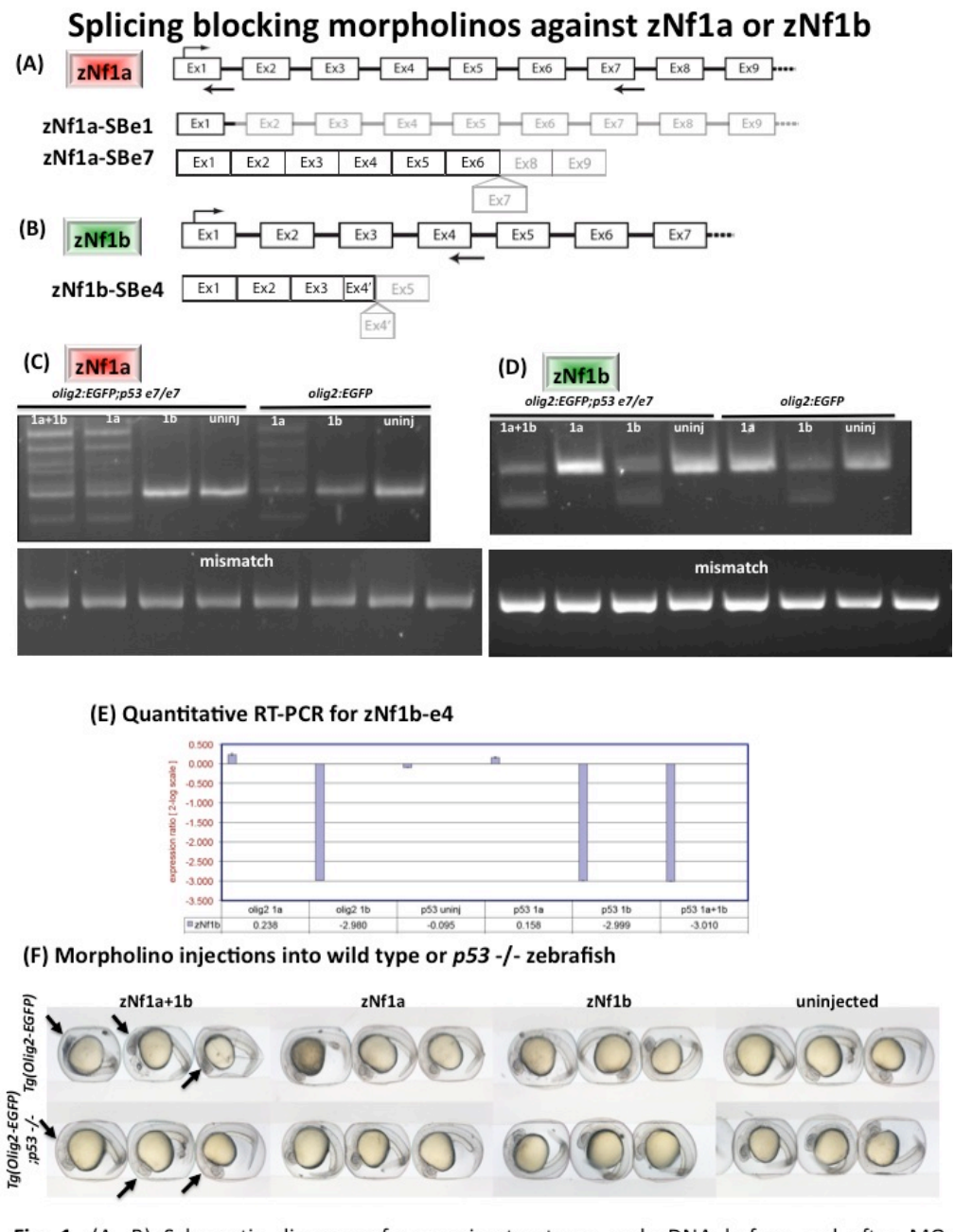
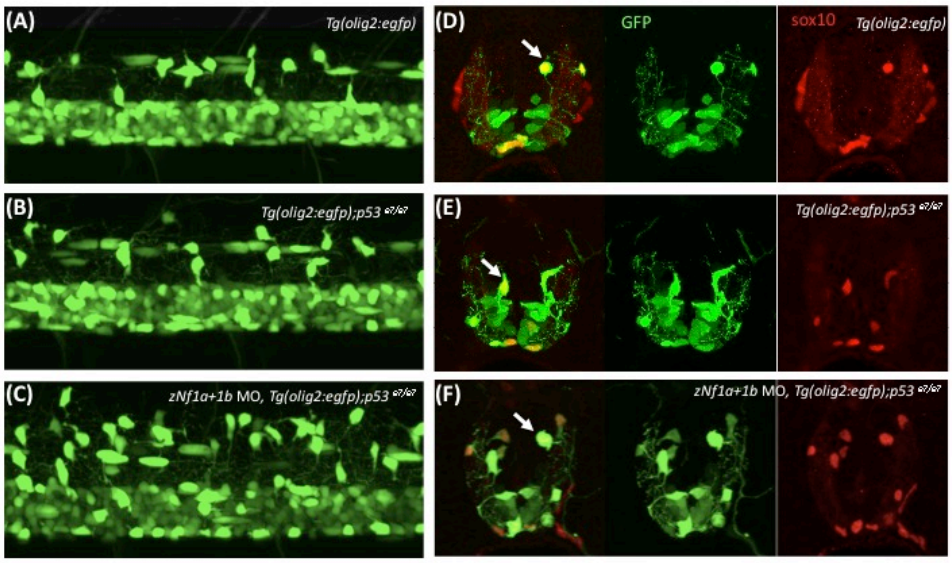


Fig. 1. (A, B) Schematic diagram of genomic structures and cDNA before and after MO knockdown of zNf1a and zNf1b. (C, D) Upper panels: RT-PCR of embryos at 3dpf injected with MOs against zNf1a or 1b alone or both into *Tg(olig2-EGFP)* or *Tg(olig2-EGFP);p53-/-* fish. Lower panel: RT-PCR of mismatch-MO injected embryos. (E) Quantitative RT-PCR for zNf1b MO–injected embryos. (F) The brain necrosis due to MO toxicity can be circumvented by utilizing *p53-/-* zebrafish. Arrows indicate necrotic or rescued brain in wild type or p53-/- background.

The number of OPCs are increased upon zNf1 knockdown



(G)

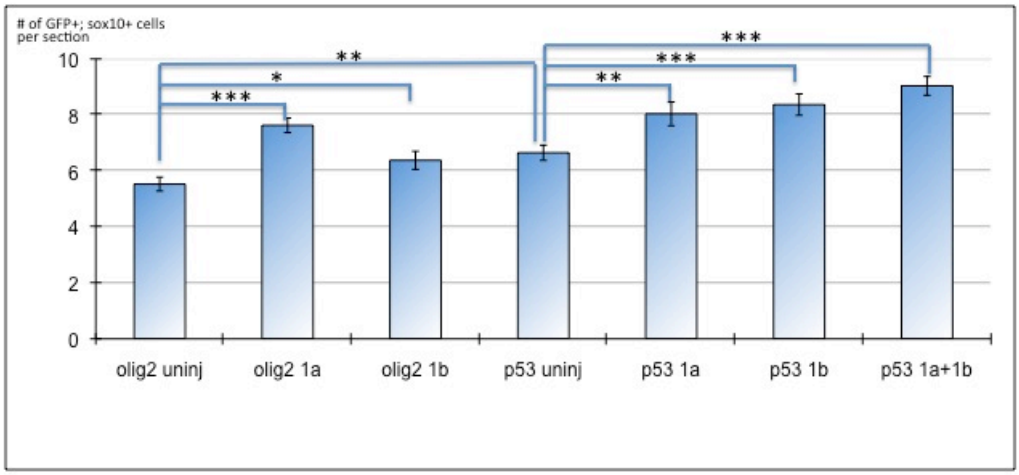


Fig. 2. (A-C) Projected confocal images of the lateral view of the spinal cord in the live *olig2-EGFP* transgenic zebrafish injected with MOs. **(D-F)** Sections of the spinal cord immunostained with sox10 antibody in red. GFP+ cells in green are olig2-positive cells from the transgenic fish. GFP+/sox10+ cells are OPCs, of which some are denoted by arrows. **(G)** The comparison and statistical analysis of the averaged numbers of GFP+/sox10+ cells per section in zNf1a (1a) or zNf1b (1b) alone or both MO-injected embryos at 3pdf. Error bar is SEM.

MAPK signaling appears to be upregulated upon zNf1 knockdown, but not in OPCs

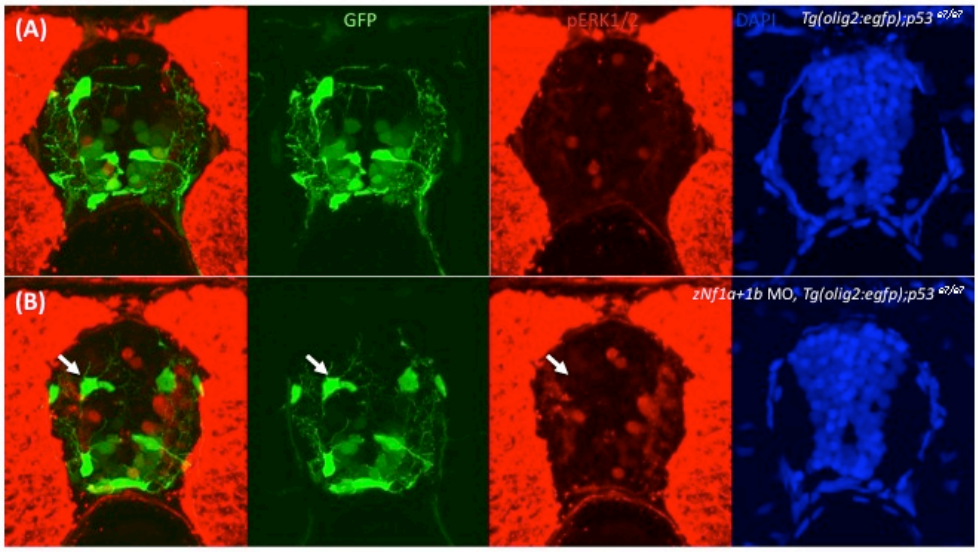


Fig. 3. Projected confocal images of the spinal cord sections immunostained with phopho ERK1/2 specific antibody in red. Green is GFP+ cells from olig2-EGFP transgenic fish. **(A)** uninjected control. **(B)** zNf1a and zNf1b MO-injected olig2-EGFP;p53-/- embryos. Arrow indicates that this OPC is GFP+, but pERK1/2-. Blue is DAPI for nuclear staining.

Cell proliferation is not evident in morphants by pH3 staining

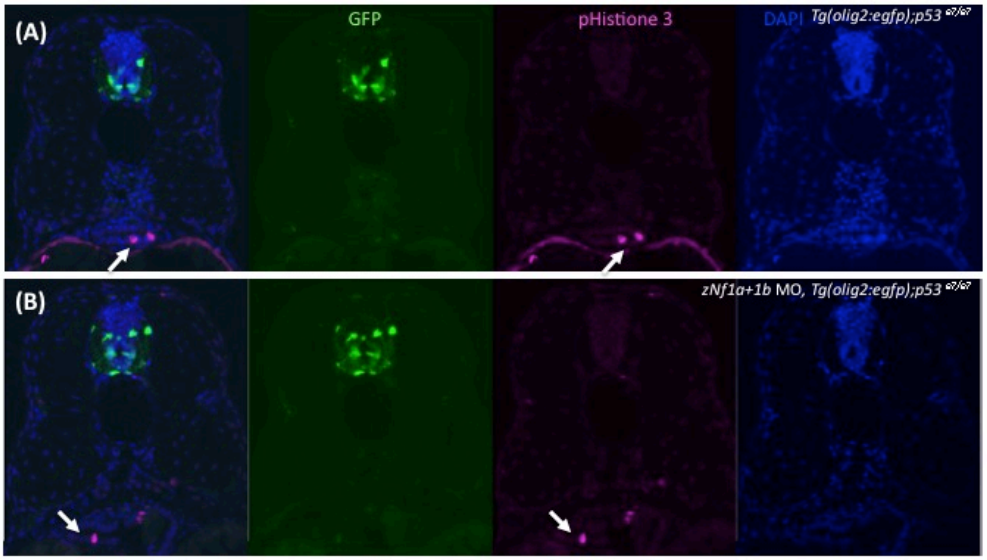
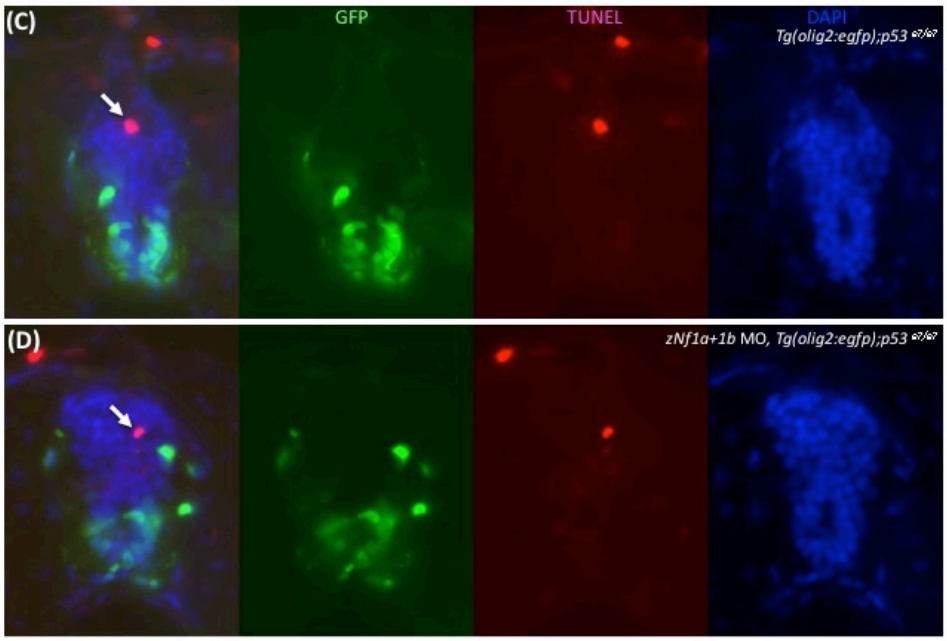


Fig. 4. Projected confocal images of the spinal cord sections immunostained with phospho-histone 3 specific antibody in magenta. Green is GFP+ cells from olig2-EGFP transgenic fish. **(A)** uninjected control. **(B)** zNf1a and zNf1b MO-injected olig2-EGFP;p53-/- embryos. Arrows indicate the proliferating cells in the gut in either condition. Blue is DAPI for nuclear staining.



(Fig. 4.) (C, D) Fluorescent images of the spinal cord sections for TUNEL (red) with GFP (green) from olig2-EGFP transgenic fish. **(C)** uninjected control. **(D)** zNf1a and zNf1b MO-injected olig2-EGFP;p53-/- embryos. Arrows indicate TUNEL+ cells in the spinal cord in either condition. Blue is DAPI for nuclear staining.

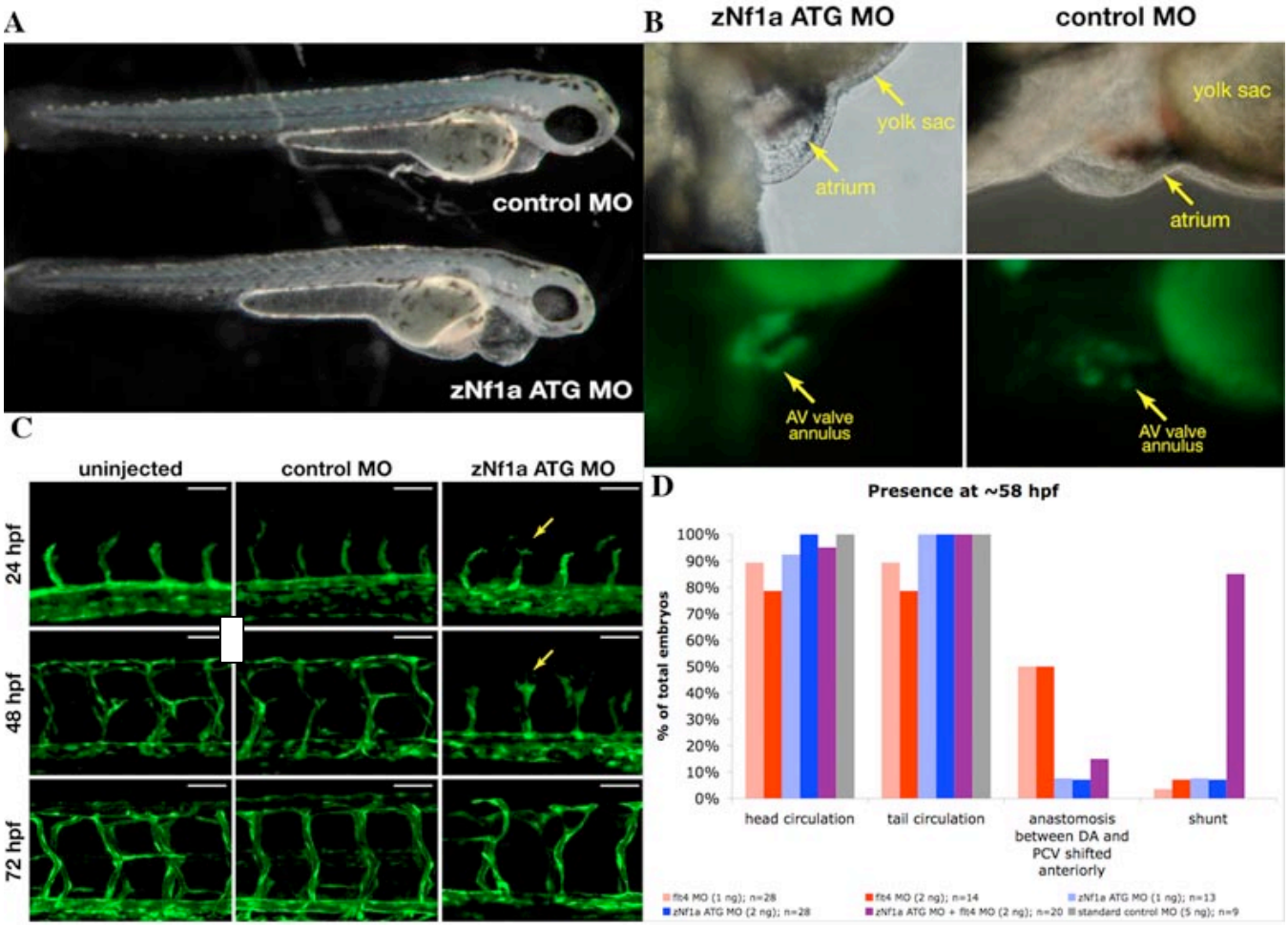
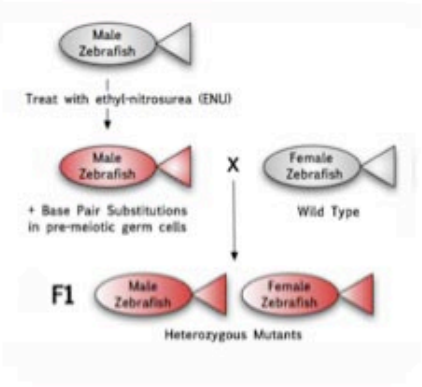


Fig. 5: Cardiovascular defects in zNf1 knockdown fish. A. Pericardial effusion produced by zNf1a MO knockdown. B. Pericardial effusion and enlarged endocardial cushions. C. Vascular patterning defects produced by zNf1a knockdown. D. Quantification of vascular patterning defects.

Searching for zebrafish mutants for zNf1a and zNf1b

(A) TILLING



(B)

	exon	mutation	Amino acid change
1 (dir. seq.)	Exon26	CAG→CAC	Gln→His
2 (74-11)	Exon26	TCA→CCA	Ser→Pro
3 (VU180)	Exon26	ATG→AAG	Met→Lys
4 (M82)	Exon21	ACG→ATG	Thr→Met
5 (F211)	Exon21	CCG→CTG	Pro→Lys

(C) retroviral insertion

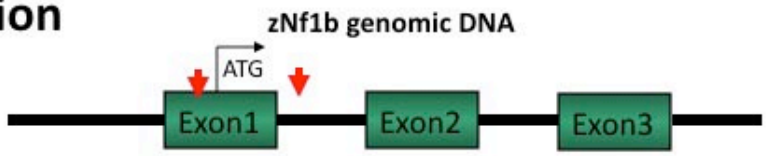


Fig. 6 **(A)** Schematics of TILLING procedure. **(B)** A list of the mutations for zNf1a identified from TILLING thus far. **(C)** Retrovirus insertions for zNf1b identified from Znomics mutant library. Red arrows indicates the positions where the insertions are located in the genomic structure of zNf1b.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterization of zebrafish orthologs: zNf1a and zNf1b.
- Analysis of gene expression of zNf1a and zNf1b.
- Analysis of morpholino knockdown phenotypes for zNf1a and zNf1b.
- Initiation of tilling experiments to identify fish with mutations in zNf1a and zNf1b.
- Obtained and began breeding fish with insertional mutations in zNf1a and zNf1b.
- Initiated experiments to mutate zNf1a and zNf1b using engineered zinc fingered nuclease.

REPORTABLE OUTCOMES:

Two manuscripts are in preparation to describe the results obtained so far.

CONCLUSION:

In this first year of study, we have made significant advances towards meeting the goals set forth in the funded proposal. The target genes have been clearly identified and significant phenotypes due to morpholino knockdown have been characterized that are relevant to the understanding of human disease. While morpholino knockdown is informative, future experiments will be aided by the characterization of stable mutations within these genes in zebrafish. Significant strides have been made towards obtaining these mutant fish lines. Several different approaches have been initiated which may result in an allelic series of mutant fish appropriate for further analysis throughout the scientific community. When characterized, these reagents will be freely shared with our colleagues. These reagents should be excellent candidates for use in screening small molecules and for use in performing genetic screens to identify modulators of this important disease.